

Effect of Environment on Sensitivity of *Neisseria gonorrhoeae* to *Pseudomonas aeruginosa* Bacteriocins

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The effect of environmental variation on the susceptibility of *Neisseria gonorrhoeae* to pyocin produced by *Pseudomonas aeruginosa* was examined. Susceptibility to at least one pyocin was demonstrated in strains of *N. gonorrhoeae* (99%), *N. meningitidis* (35%), and *N. lactamica* (47%). The degree of sensitivity to pyocin displayed by *N. gonorrhoeae* was affected by varying the pH of the growth environment. Gonococcal strains were more sensitive to growth inhibition by pyocins at an alkaline pH and less sensitive to growth inhibition at an acid pH. Inhibitory titers fluctuated during nonselective subculture of fresh clinical isolates. There was no apparent correlation between auxotype and sensitivity to pyocin. Also, no relationship between colony morphology and pyocin sensitivity was seen.

Pyocins (R-type bacteriocins isolated from *Pseudomonas aeruginosa*) morphologically resemble tail fibers of phage (11) and inhibit the growth of *Neisseria gonorrhoeae* (17, 23). Because of the differential effect of pyocins on pathogenic and nonpathogenic species of *Neisseria*, Morse et al. (17) suggested that inhibition of growth by pyocins may be used as a typing system. One prerequisite of a typing system is that the reactive surface components remain stable during initial isolation and subsequent subculture of the microbes. *N. gonorrhoeae* is an organism that can modify its cellular surface in response to environmental conditions. For example, when grown in vitro it differs morphologically and antigenically from cells seen in urethral exudates and from cells grown in vivo (18, 19). Kellogg (13) observed that colony types T₁ and T₂ undergo a rapid shift to colony types T₃ and T₄ when subcultured nonselectively. Novotny et al. (18) noted that the envelope of cells grown in vivo differs markedly in appearance from cells grown in vitro. Furthermore, the gonococcus can grow over the range of pH 5.8 to pH 8.3 (optimum growth at pH 7.3) (1, 14). Brookes and Hedén (1) have shown that the greatest cell yield is obtained at pH 6.4. The amount of peptidoglycan and the extent of associated protein varies considerably depending upon the pH of the growth medium (6-8).

Since *N. gonorrhoeae* can readily change its cell surface if its environment is changed, this investigation was undertaken to explore the effect of environmental changes on pyocin sensitivity. The following parameters were examined: (i) effect of repeated subculture on fresh isolates; (ii) influence of growth pH; (iii) correlation of

pyocin sensitivity with nutritional requirements; (iv) correlation of pyocin sensitivity with trypsin sensitivity; and (v) correlation of pyocin sensitivity with transparent and opaque colony types. The data presented establish that pyocin sensitivity varies significantly during propagation of gonococci grown under different environmental conditions.

MATERIALS AND METHODS

Organisms. Isolates of *N. gonorrhoeae*, *Neisseria meningitidis*, *Moraxella* spp., and nonpathogenic *Neisseria* used in this study were obtained from the diagnostic laboratories at Strong Memorial Hospital, Rochester, N.Y. All pathogenic isolates were initially propagated on JEMBEC plates (Flow Laboratories). Strains of pathogenic and nonpathogenic *Neisseria* were also provided by S. A. Morse (University of Oregon Health Science Center, Portland). Strains of *N. meningitidis* type B were obtained from H. Feldman (State University of New York, Syracuse). Species of *Acinetobacter* and *Moraxella* were kindly supplied by B. W. Catlin (Medical College of Wisconsin, Milwaukee). *N. gonorrhoeae* JW31 and the pyocin 103-resistant strains JW31pyR1, JW31pyR2, and JW31pyR3 were also obtained from S. A. Morse. Strain JW31pyR4 was derived from JW31 by selection for resistance to pyocin 103. Other strains of *N. gonorrhoeae* were obtained from a collection of 500 strains provided by the Center for Disease Control, Atlanta, Ga. The identity of all organisms was confirmed by Gram stain, oxidase reaction, and the production of acid from specific carbohydrates.

Pyocinogenic strains of *Pseudomonas aeruginosa*, FS1, FS2, FS3, FS4, FS5, FS8, FS18, and PA103, as well as the indicator strain PS-7, were obtained from S. A. Morse. In this study pyocins isolated from the above pyocinogenic strains will be designated 1, 2, 3, 4, 5, 8, 18, and 103, respectively.

Growth conditions and media. All species of

Neisseria, *Acinetobacter*, and *Moraxella* were grown in liquid medium (GCBI) containing (grams per liter): proteose peptone no. 3 (Difco), 15; soluble starch (Difco), 1; K_2HPO_4 , 4; KH_2PO_4 , 1; and NaCl, 5. The medium was supplemented before use with 0.042% $NaHCO_3$ and 1% IsoVitalX (BBL Microbiology Systems). To produce pyocins, *P. aeruginosa* was grown in the GCBI supplemented with glycerol (1%, vol/vol) and monosodium glutamate (8.46 g/liter).

Solid medium (GCI) was prepared by the addition of agar (20 g/liter; Difco) to GCBI. When required, the pH was adjusted to pH 6.5, 7.2, or 8.0 with 6 N NaOH or 6 N HCl before autoclaving.

Induction and purification of R-type pyocins.

An overnight culture of a pyocinogenic strain of *P. aeruginosa* (10 ml) was centrifuged ($10,000 \times g$, 10 min, 4°C) under aseptic conditions. The suspended pellet was inoculated into fresh medium (500 ml), incubated at 37°C, and aerated by shaking (New Brunswick Scientific, model G76). The turbidity was monitored by a Klett-Summerson Colorimeter (filter no. 66). At a turbidity of 150 Klett units, mitomycin C was added (final concentration, 1 µg/ml). Incubation was continued (250 rpm, 37°C) until extensive lysis was observed. After the addition of chloroform (5%, vol/vol), the suspension was allowed to stand overnight at 4°C. The suspensions were centrifuged ($4,000 \times g$, 30 min, 4°C) to remove cellular debris.

Pyocins were purified by a modification (17) of the method of Kageyama and Egami (12). Briefly, 30 ml of 1 M $MnCl_2$ was added per liter of lysate, and the pH was adjusted to 7.5 by the addition of 6 N NaOH. After centrifugation, $(NH_4)_2SO_4$ was added to a final concentration of 70%. Preparations were centrifuged, and the pellets were suspended in buffer (0.5 M Tris, pH 7.5; 0.5 M $MgCl_2$; 0.5 M $MgSO_4$) and then dialyzed overnight against 2 liters of the same buffer. The partially purified pyocin preparations were sterilized by passage through a 0.45-µm filter (Nalgene).

Assay of pyocin activity. Since pyocin preparations 4, 5, 8, and 18 were incapable of inhibiting the growth of *P. aeruginosa* indicator strain PS-7, they were standardized by using *N. gonorrhoeae* Y-17B as an indicator strain. This was considered an acceptable procedure because similar values for pyocin 1, 2, 3, and 103 titers were obtained with *N. gonorrhoeae* Y-17B and *P. aeruginosa* PS-7 for those pyocins which killed both of these strains.

Standardized pyocin preparations containing ca. 700 U/ml were serially diluted. A 1.5-µl aliquot of each dilution was spotted onto a lawn of test cells. The lawn of cells was prepared by suspending cells grown overnight on GCI in 0.85% saline. The turbidity was adjusted to 50 Klett units, and 0.1 ml was streaked onto a GCI plate. One unit of pyocin activity was defined as the minimal concentration of pyocin that gave a clear zone of inhibition when spotted on a lawn of 10^8 colony-forming units. The titer was defined as the minimal concentration of pyocin required to give 1 unit of pyocin activity.

Nonselective subculture. Fresh clinical isolates were cloned on a GCI plate. Strains were subcultured daily for a period of 10 days. Pyocin sensitivity patterns were determined after every other subculture.

RESULTS

Spectrum of pyocin activity. Morse et al. (17) reported that most strains of *N. gonorrhoeae* and a few strains of *N. meningitidis* and *Neisseria lactamica* were sensitive to growth inhibition by pyocin 103. This pyocin was not active against other *Neisseria* spp. We tested several *Neisseria* species for sensitivity to eight different pyocins (Table 1) and found that 99% of the strains of *N. gonorrhoeae*, 35% of the strains of *N. meningitidis*, and 47% of the *N. lactamica* strains were inhibited by at least one of the eight pyocins. No other species of *Neisseriaceae* were inhibited by pyocin. Of the 116 strains of *N. meningitidis* tested, 62 were serogroup type B isolated from patients suffering from meningitis; 21 of these strains were sensitive to pyocin. Forty strains were isolated as normal flora with a serotype of one of the other classes; 13 of these strains were sensitive to pyocin. Fourteen strains were nontypable; six of these were sensitive to pyocin.

Relationship of auxotype to pyocin sensitivity. Because of the possible effects of nutritional requirements on the expression of all envelope components, strains of various auxotypes were assayed for sensitivity to growth inhibition by pyocin. Although a variety of pyocin sensitivity patterns were detected, the data (Table 2) indicate no apparent relationship between auxotype and a particular pyocin sensitivity pattern.

Effect of pH on pyocin sensitivity. Since changes in the cell surface of *N. gonorrhoeae* occur as a function of the pH of the growth medium (6), the effect of growth pH on the pyocin sensitivity of the gonococcus was explored. Lawns of *N. gonorrhoeae* were prepared on GCI agar adjusted to pH 6.5 or 8.0, and 1.5-

TABLE 1. Inhibition of *Neisseriaceae* by at least one of eight different test pyocins^a

Organism	No. of isolates		% Sensitive
	Tested	Sensitive	
<i>N. gonorrhoeae</i>	95	94	99
<i>N. meningitidis</i>	116	40	35
<i>N. lactamica</i>	15	7	47
Other species of <i>Neisseria</i> ^b	30	0	0
<i>Acinetobacter</i> spp.	3	0	0
<i>Moraxella</i> spp.	6	0	0
<i>Branhamella catarrhalis</i>	3	0	0

^a Sensitivity was determined as described in the text.

^b Other *Neisseria* spp. group is composed of 4 strains of *N. flava*, 4 strains of *N. sicca*, and 22 strains of oxidase-positive, gram-negative diplococci which fermented glucose, maltose, sucrose, and fructose.

TABLE 2. Relationship of gonococcal auxotype to pyocin sensitivity^a

Inhibition by pyocin:									No. of strains with auxotype ^b :					Percent of total
1	2	3	4	5	8	18	103	Prototroph	Pro ⁻	Arg ⁻	Arg ⁻ Hyx ⁻ Ura ⁻	Other		
+	+	+	-	+	+	-	+	24	12	10	8	6	39.7	
+	+	+	+	+	+	+	+	17	4	0	8	4	17.2	
+	-	+	-	-	-	-	+	16	1	2	0	1	13.2	
+	+	+	-	+	+	+	+	12	0	0	1	0	8.6	
-	-	-	-	-	-	-	+	2	2	2	2	2	6.6	
+	+	+	+	+	+	-	+	1	0	0	0	3	2.6	
+	-	+	-	-	+	-	+	1	2	0	1	0	2.6	
+	+	+	-	-	-	-	+	0	2	0	0	2	2.6	
+	+	+	-	-	+	-	+	0	0	0	0	3	2.0	
+	+	+	-	+	-	-	+	0	0	0	0	3	2.0	
+	-	+	+	+	+	+	+	1	0	0	0	0	0.7	
+	-	+	-	+	+	+	+	1	0	0	0	0	0.7	
-	-	+	-	+	-	-	+	0	0	0	0	1	0.7	
-	-	-	-	+	-	-	+	0	0	0	0	1	0.7	
-	-	-	-	-	-	-	-	0	1	0	0	0	0.7	

^a Determination of pyocin sensitivity as described in the text.

^b Determination of auxotype was as previously described (20). Since all gonococci require cysteine for growth, these strains are designated prototroph. Arg⁻, Hyx⁻, Ura⁻, and Pro⁻ refer to the nutritional requirements for arginine, hypoxanthine, uracil, and proline, respectively.

μl samples of pyocin (700 U/ml) were added. The sensitivity of strains to a serial twofold dilution of pyocin is shown in Fig. 1. The dotted line is the theoretical line that would be described if there were no difference between the sensitivity at pH 6.5 and 8.0. Pyocin 103 displayed the highest degree of stability with respect to its ability to inhibit the growth of *N. gonorrhoeae*. Pyocin 1 showed some variability in its ability to inhibit the growth of the test organisms, and pyocins 5 and 8 showed a large fluctuation, with strains sensitive to killing at pH 8.0 and resistant to killing at pH 6.5. Pyocin 3 showed results similar to those of pyocin 1, whereas pyocins 4 and 18 showed results similar to pyocins 5 and 8. The control, *P. aeruginosa* PS-7, showed no change in inhibition over the pH range tested (data not shown).

Effect of nonselective transfer of *N. gonorrhoeae* on pyocin sensitivity. During nonselective transfer of *N. gonorrhoeae*, changes in colonial morphology occur which are often accompanied by a loss of pili (13, 23). Because of the possible effect of piliation on pyocin sensitivity, a preliminary test was performed using different colony types of *N. gonorrhoeae* strains F62 and RUG38. There was no difference in the sensitivity of these strains as a function of colony type (data not shown). Therefore, no attempt was made to maintain a particular colony type in subsequent studies.

To examine the effect of nonselective transfer of gonococci on pyocin sensitivity, fresh clinical isolates were subcultured daily onto GCI plates

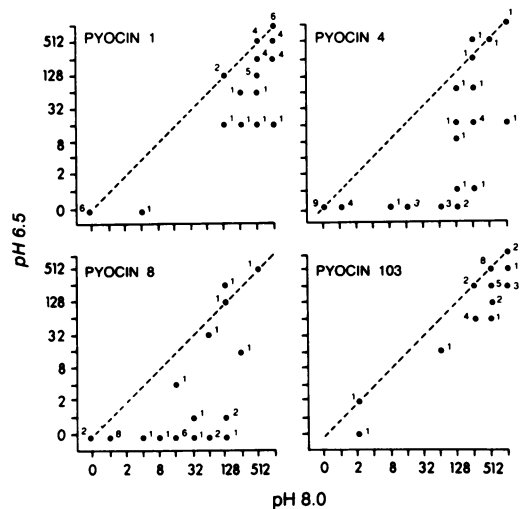


FIG. 1. Variation of pyocin inhibition at various pH values. Numbers on axes represent inhibitory titers. Dashed line is what is expected if there is no titer change when inhibitory titers seen at pH 6.5 are plotted against inhibitory titers seen at pH 8.0. Numbers next to points indicate the number of strains which that point represents.

for 10 days. The pyocin sensitivities of these strains were titrated every other day. After 5 days of nonselective transfer, the colony morphology was entirely type 4. Strains sensitive to pyocins 103, 1, and 3 showed little variation in inhibitory titer to these pyocins when tested at the different days. In contrast, strains sensitive

to pyocins 2, 4, 5, 8, and 18 changed markedly in inhibitory titer over the test period (Fig. 2). There was no predictable shift in sensitivity to any of the pyocins as a function of the number of subcultures. Some gonococcal strains varied in sensitivity during subculture. For example, after a few passages some strains became resistant and then reverted to sensitivity after further subculturing. By contrast, the *Pseudomonas* control PS-7 showed no change in titer over the test period.

Correlation of pyocin sensitivity with colony morphology and trypsin sensitivity. Isogenic strains of *N. gonorrhoeae* with the phenotypic differences in opaque and transparent colony types were assayed for sensitivity to the eight test pyocins. No differences were seen with respect to pyocin sensitivity patterns (Table 3), when tested on Swanson's medium (22) or GCI.

Because trypsin can affect the growth of *N. gonorrhoeae*, trypsin-sensitive and trypsin-resistant strains of *N. gonorrhoeae* were tested to examine a possible relationship between the two phenotypes. Preliminary studies with 14 strains

of *N. gonorrhoeae* indicate that pyocin sensitivity patterns of trypsin-sensitive and trypsin-resistant gonococci are similar.

DISCUSSION

This study has established that: (i) pathogenic strains of *Neisseriaceae* are sensitive to pyocin; (ii) most gonococcal isolates showed only one of a few patterns of sensitivity to the eight types of pyocin tested; (iii) the sensitivity to pyocin was dependent upon the pH of the growth medium, with cells grown at pH 8.0 being more sensitive to growth inhibition by pyocin than when they were grown at pH 6.5; (iv) the sensitivity to pyocin was not stable during repeated subculturing of isolates; (v) the auxotype of the gonococcal strain had no correlation with its pyocin sensitivity pattern; (vi) there was no difference between phenotypic variants of colony morphology.

The fluctuation in pyocin sensitivity shown by the gonococci in response to environmental conditions could be due to a decrease in the quantity and availability of the pyocin receptors. Lipopolysaccharide has been implicated as the receptor for R-type bacteriocins in *P. aeruginosa* (2-4, 9, 10), but the specific surface components necessary for pyocin receptor activity in the gonococcus are unknown. *N. gonorrhoeae* cells grown at lower pH values have been reported to metabolize glucose differently from cells grown at pH 7.2 (5, 14-16) and to produce an increase in the amount of peptidoglycan and peptidoglycan-associated protein(s) (6-8). Growth at lower pH also resulted in the incorporation of more carbon from glucose into cell envelope components (15). These observations demonstrate an intimate relationship between environmental parameters and cell surface components in *Neisseriaceae*. If the hypothesis that lipopolysaccharide serves as the receptor for pyocin is correct, then those pyocins which inhibit the growth of most species of *Neisseria* could have recognition sites located in more stable regions of the lipopolysaccharide. Conversely, those pyocins that demonstrated varying degrees of growth inhibition could have receptors situated on regions of the lipopolysaccharide subject to greater modification as the gonococci responded to the growth environment. Therefore, the fluctuation in pyocin titers may be a reflection of the amount or composition of O-polysaccharide (O-antigen side chains) that is attached to the core region. Such changes in polysaccharide components may be related to selective degradation of the O-polysaccharides or due to substitution of sugars within the O-antigen side chain. Alternatively, the core region could serve as the receptor.

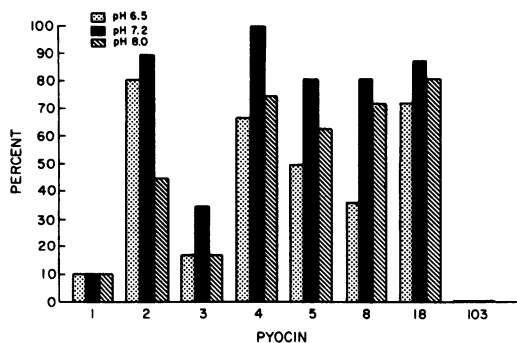


FIG. 2. Effect of nonselective transfer of *N. gonorrhoeae* on pyocin sensitivity. Percent is the number of strains sensitive to pyocin which showed a fourfold change in sensitivity over the 10-day test period, divided by the total number of sensitive strains tested.

TABLE 3. Pyocin sensitivity of transparent and opaque colonies^a

Strain ^b	Pyocins affecting colony type ^c :	
	Transparent	Opaque
RUG 1208	1, 3, 103	1, 3, 103
RUG 1004	1, 2, 3, 5, 8, 103	1, 2, 3, 5, 8, 103
RUG 1232	1, 2, 3, 5, 8, 103	1, 2, 3, 5, 8, 103
RUG 1115	1, 2, 3, 5, 8, 103	1, 2, 3, 5, 8, 103
F 62	1, 2, 3, 5, 8, 18, 103	1, 2, 3, 5, 8, 18, 103

^a Opaque and transparent colony types were determined as described by Swanson (22).

^b Isogenic strains showing phenotypic differences of transparent and opaque colony morphology were tested.

^c Numbers indicate sensitivity to particular pyocin. Sensitivities were determined as described in the text.

A gonococcal typing system based on pyocin sensitivity has been recently proposed and is currently under investigation (17, 21; J. Borst and J. H. L. DeJong, Abstr. Antonie van Leeuwenhoek 44:254, 1978). Sidberry and Sadoff (21) have reported stability in pyocin sensitivity patterns among gonococcal strains cultured 4 months apart. The data also suggest that growth inhibition by pyocin was stable exclusive of the site of isolation within the host. Matching pyocin patterns were also reported between consorts infected with gonorrhoea (21). However, in a more recent study, Borst and DeJong (Abstr. Antonie van Leeuwenhoek 44:254, 1978) have shown that identical patterns of pyocin sensitivity were revealed in only 61% of the cases. Nevertheless, the consistent change in patterns coupled with epidemiological analysis indicated that "an epidemiological useful conclusion" was possible in 89% of the cases. In view of the substantial variability of *N. gonorrhoeae* in response to environmental growth conditions observed in our study, we suggest that the development of a diagnostic procedure based on pyocin sensitivity be approached cautiously. The analysis of pyocin sensitivity may prove to be a more important tool in the study of alterations on the cell surface.

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